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<b>(54) Title:</b> RECOMBINANT HEREGULINS AND THEIR BIOLOGICAL FUNCTIONS UPON RECEPTOR ACTIVATION  <b>(57) Abstract</b>  Disclosed are generation of versatile recombinant heregulins and some of the biological functions and intracellular signaling pathways that these proteins trigger following receptor activation. Also disclosed is the cloning of the cDNA fragments encoding the EGF-like domain of HRG- $\alpha$ , - $\beta$ 2 or - $\beta$ 3 into an eukaryotic expression vector and the transfection of the vector into mammalian cells. The recombinant heregulins are useful in activating the HER4 receptor.		

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## RECOMBINANT HEREGULINS AND THEIR BIOLOGICAL FUNCTIONS UPON RECEPTOR ACTIVATION

5           The present invention relates to the generation of versatile recombinant heregulins (HRGs) and some of the biological functions and intracellular signaling pathways that these proteins trigger following receptor activation.

          Heregulins (HRGs) are mosaic glycoproteins that bind  
10 to, and induce tyrosine phosphorylation of the HER4/P180<sup>erbB4</sup> receptor. Heregulins (HRGs), Holmes, W. E. et al., (1992), Science 256, 1205-1210, neu differentiation factor (NDF), Peles, E. et al., (1992), Cell 69, 205-216; Wen, D. et al., (1992), Cell 69, 559-572 and Wen, D. et al., (1994), Mol. Cell Biol. 14, 1909-1919,  
15 glial growth factors (GGFs), Marchionni, M. A. et al., (1993) Nature 362, 312-318, and acetylcholine receptor-inducing activity (ARIA), Falls, D. L. et al., (1993) Cell 72, 801-815, are homologous multifunctional proteins. The HRG isoforms originate from a single gene by alternative RNA splicing. HRG cDNAs encode  
20 large transmembrane precursors with multiple domains including an immunoglobulin-like domain, a spacer domain with several glycosylation sites, an EGF-like domain, a juxtamembrane domain of variable length, a transmembrane region, and a cytoplasmic domain. The soluble mature HRGs are released from the cell  
25 surface by proteolytic cleavage. GGFs, Marchionni, M. A. et al., (1993) Nature 362, 312-318, and ARIA; Falls, D. L. et al., (1993) Cell 72, 801-815, which were isolated from brain tissues, also contain a kringle-like domain that is absent in HRGs and NDFs.  $\alpha$

and  $\beta$  HRG isoforms display sequence differences in the third loop of the EGF-like domain, and the juxtamembrane domain. The EGF-like domain of HRGs contains six cysteine residues that are characteristic of the EGF family of growth factors including EGF,

5 Carpenter, G. et al., (1979) Ann. Rev. Biochem. 48, 193-216, transforming growth factor- $\alpha$ , Derynck, R. et al., (1984) Cell 38, 287-297, vaccinia virus growth factor, Blomquist, C. I. et al., (1984) Proc. Natl. Acad. Sci. USA 81, 7363-7367, amphiregulin, Shoyab, M. et al., (1989) Science 243, 1074-1076, heparin-binding EGF-

10 like growth factor, Higashiyama, S. et al., (1991) Science 251, 936-939, and betacellulin, Shing, Y. et al., (1993) Science 259, 1604-1607.

Although HRGs contain an EGF-like motif, they do not bind to EGFR/P170<sup>erbB1</sup>, Holmes, W. E. et al., (1992), Science 256,

15 1205-1210. The HRGs bind to HER4/p180<sup>erbB4</sup>, a recently isolated member of the epidermal growth factor receptor family, Plowman, G. D. et al., (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 1746-1750; Culouscou, J. M. et al., (1993) J. Biol. Chem. 268, 18407-18410 and Plowman, G. D. et al., (1993) Nature (London)

20 366, 473-475. The HER3/P180<sup>erbB3</sup> receptor, another member of this family has also been reported to be a receptor for HRGs, Carraway, K. L. et al., (1994) J. Biol. Chem. 269, 14303-14306. HRGs do not directly interact with HER2/p185<sup>erbB2</sup> receptor, as originally proposed, Holmes, W. E. et al., (1992), Science 256,

25 1205-1210 and Peles, E. et al., (1992), Cell 69, 205-216. The HER2/p185<sup>erbB2</sup> indirectly participates in HRG-mediated signaling through transphosphorylation or receptor heterodimerization with HER4 and/or HER3, Plowman, G. D. et al.,

(1993) Nature (London) 366, 473-475 and Carraway, K. L. et al., (1994) J. Biol. Chem. 269, 14303-14306.

As a consequence of HRG/NDF binding to its receptors, human mammary tumor cells have been shown to differentiate,  
5 Peles, E. et al., (1992), Cell 69, 205-216 and Bacus, S. S. et al., (1993), Cancer Res. 53, 5251-5261, and up-regulate their expression of the intercellular adhesion molecule-1 (ICAM-1), Bacus, S. S. et al., (1993), Cancer Res. 53, 5251-5261. The biological effects of HRGs are mediated through receptors that  
10 possess an intrinsic tyrosine kinase activity and are autophosphorylated upon HRG binding, Plowman, G. D. et al., (1993) Nature (London) 366, 473-475. The studies carried out on receptor tyrosine kinases such as the epidermal growth factor receptor, the platelet-derived growth factor receptor and the insulin  
15 receptor, have demonstrated a crucial role for receptor autophosphorylation in intracellular signal transduction following ligand binding, Ullrich, A. et al., (1990) Cell 61, 203-212 and White, M. F. et al., (1994) J. Biol. Chem. 269, 1-4. It has been demonstrated that specific autophosphorylation sites on receptor  
20 tyrosine kinases serve as recognition structures for target molecules containing Src homology 2(SH2) domains. SH2 domains are conserved noncatalytic sequences of approximately 100 amino acid found in various signaling molecules and oncogenic proteins, Koch, C. A. et al., (1991) Science 252, 668-  
25 674 and Songyang, Z. et al., (1993) Cell 72, 767-778. SH2 domain-containing proteins bind with high affinity to phosphotyrosine residues in the context of specific flanking amino acids. For example, the p85 subunit of phosphatidylinositol (P

l)3'-kinase (PI 3-K), the p21ras GTPase activating protein (GAP), and phospholipase C $\gamma$ (PLC- $\gamma$ ) have been shown to contain SH2 domains. More recently, SH2 domain-containing proteins that lack an apparent catalytic domain and seem to function as

5 adaptors linking proteins involved in signal transduction have been described, Lowenstein, E. J. et al., (1992) Cell 70, 431-442; Pelicci, G. et al., (1992) Cell 70, 93-104; Egan, S. E. et al., (1993) Nature (London) 363, 45-51; Skolnik, E. Y. et al., (1993) Science 260, 1953-1955; Rozakis-Adcock, M. et al., (1993) Nature

10 (London) 363, 83-85 and Gale, N. W. et al., (1993) Nature (London) 363, 88-92. One of them, Shc was identified and cloned based on its homology to SH2 sequences from the human c-fes gene, Pelicci, G. et al., (1992) Cell 70, 93-104. The Shc cDNA is predicted to encode two proteins of 46 and 52 kDa that contain a

15 single C-terminal SH2 domain and a collagen-homologous region that is rich in glycine and proline. No catalytic domain was identified in Shc. Anti-Shc antibodies have been shown to recognize three proteins of 46, 52, and 66 kDa in a wide range of mammalian cells. A variety of growth factors and cytokines have

20 been shown to induce phosphorylation of Shc proteins, Pronk, G. J. et al., (1993) J. Biol. Chem. 268, 5748-5753; Yokote, K. et al., (1994) J. Biol. Chem. 269, 15337-15343; Schorb, W. et al., (1994), J. Biol. Chem. 269, 19626-19632; Cutler, R. L. et al., (1993) J. Biol. Chem. 268, 21463-21465; Burns, L. A. et al., (1993) J. Biol. Chem.

25 268, 17659-17661 and Damen, J. E. et al., (1993) Blood 82,2296-2303. The overexpression of the Shc proteins is associated with a transformed phenotype in fibroblasts, Pelicci, G. et al., (1992) Cell 70, 93-104, and neuronal differentiation of PC12 cells, Rozakis-

Adcock, M. et al., (1992) Nature (London) 360, 689-692, strongly suggesting that Shc is involved in cell growth regulation.

The present invention is directed to the generation of versatile recombinant heregulins (HRGs) and the biological functions and intracellular signaling pathways that these proteins trigger following receptor activation.

Because the EGF-like domain of HRG is sufficient for receptor binding, the present invention relates to the cloning of the cDNA fragments encoding the EGF-like domains of HRG- $\alpha$ , - $\beta$ 2, or - $\beta$ 3 into an eukaryotic expression vector containing sequences encoding a thrombin cleavage site, followed by the Fc portion of a human IgG1. The present invention also relates to the production of the recombinant fusion proteins rHRGs-T-Fc which can be used as chimeric proteins or as EGF-like domains (reHRGs) after thrombin cleavage and removal of the Fc portion of the molecule.

The present invention demonstrates that the recombinant HRGs, in either form bind to and activate the HER4 receptor and that the Shc proteins are tyrosine phosphorylated following HRG stimulation. The present invention also demonstrates that rHRG- $\alpha$ -T-Fc bound to human breast cancer cells that express HER4 receptors and induced the expression of the intercellular adhesion molecule-1. Moreover, reHRG- $\beta$ 2 markedly induced phosphorylation of Shc proteins on tyrosine, suggesting a role for these adaptor molecules in HRG-mediated signaling.

Figure 1 illustrates tyrosine autophosphorylation of the HER4 receptor following rHRGs-T-Fc stimulation.

Figure 2 illustrates the binding of rHRG- $\alpha$ -T-Fc to MDA-MB-453 cells.

Figure 3 illustrates the induction of ICAM-1 expression in response to rHRG- $\alpha$ -T-Fc.

5 Figure 4 illustrates thrombin cleavage of rHRG- $\beta$ 3-T-Fc.

Figure 5 illustrates the stimulation of protein phosphorylation in response to reHRGs.

Figure 6 illustrates tyrosine phosphorylation of Shc proteins upon HER4 activation.

10 Figure 7 shows the nucleotide and amino acid sequences (SEQ. ID. NOS. 8 and 9) of a heregulin alpha fusion protein. In this particular protein, the nucleotide bases correspond to the following:

	<u>Bases</u>	<u>Region</u>
15	1 to 178	CD5 signal sequence
	179 to 373	Heregulin alpha EGF-like binding domain
	374-424	Thrombin cleavage site
	425 to 1129	Human Ig-constant region

20 The present invention is directed to the generation of recombinant EGF-like domains of HRGs and the biological effects induced by them as well as identifying intracellular molecules involved in HER4 signaling. In preferred embodiments, the present invention relates to the cloning of the EGF-like domains of  
 25 HRG- $\alpha$ , - $\beta$ 2 and - $\beta$ 3 into an eukaryotic expression vector in frame with sequences encoding a thrombin cleavage site followed by the Fc portion of a human IgG1. More preferably, the vector further comprises the signal sequence of the CD5 protein which allows



efficient processing and secretion of the proteins. The vector is a mammalian expression vector.

The present invention relates to the creation of chimeric genes which direct the expression of recombinant fusion proteins, rHRGs-T-Fc. The recombinant fusion proteins are expressed in large amounts by transfecting the vector onto a suitable host. The preferred host is mammalian COS cells. These proteins are shown to stimulate the phosphorylation of HER4/P180<sup>erbB4</sup>. The bivalent fusion proteins generated are useful as growth factors since they activate growth factor receptors. These fusion proteins are also useful in being detected like antibodies, via their Fc domain.

The recombinant fusion proteins can also be used in high throughput screening assay for identifying low molecular weight agonist or antagonist of HER3 and HER4 receptors. The high throughput screening assay involves screening several hundreds of compounds in a short period of time in microliter well plates using reagents. The assay is carried out with the assistance of robotics and automation. The assay could be a binding assay or an enzyme assay, etc., depending on the compound being screened.

The present invention is also useful for the production of large amounts of other recombinant growth factors, such as epidermal growth factor, transforming growth factor-alpha, amphiregulin, betacellulin, heparin-binding epidermal growth factor, vaccinia growth factor, cripto, insulin growth factor, insulin-like growth factor, transforming growth factor-beta, platelet-derived growth factor, fibroblast growth factor, and nerve growth factor.

The present invention also relates to purified EGF-like domains (reHRGs) after thrombin protease cleavage of the rHRGs-T-Fc fusion proteins. These reHRGs are shown to stimulate protein phosphorylation in HER4 expressing cells.

5           The present invention is also directed to a method of purifying the fusion proteins rHRGs-T-Fc in a single step by protein A-Sepharose chromatography.

          The present invention demonstrates that reHRG- $\beta$ 2 markedly induces phosphorylation of Shc proteins on tyrosine.  
10       This suggests a role for these adaptor molecules in HRG-mediated signaling.

          Technical terms used throughout this application are well known to those skilled in the art of molecular genetics. Definition of these terms are found in many textbooks dedicated to  
15       the molecular biology field, such as "Genes," Second Edition, by Dr. Benjamin Lewis, 1985, John Wiley & Sons, Inc., New York. Abbreviations utilized in this invention are defined below:

List of Abbreviations

20	HRG	heregulin
	EGF	epidermal growth factor
	EGFR	EGF receptor
	HER	Human EGF receptor
	NDF	neu differentiation factor
25	ARIA	acetylcholine receptor
		inducing activity
	GGF	glial growth factor
	p185erbB2	HER2 encoded protein

	p180erbB4	HER4 encoded protein
	ICAM-1	intercellular adhesion molecule-1
	SH2	Src homology 2
5	CHO	Chinese hamster ovary
	SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
10	Bes	N, N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid
	PBS	phosphate-buffered saline.

15 The construction of the rHRGs-T-Fc fusion proteins to generate versatile recombinant HRGs for studying the various aspects of the biology of the HER4/HRG receptor/ligand pair is as follows: since the EGF-like domain of HRG- $\beta$ 1 had previously been shown to be sufficient for receptor binding, Holmes, W. E. et al., (1992), Science 256, 1205-1210, three chimeric genes are

20 constructed that encode soluble proteins consisting of the EGF-like domain of HRG- $\alpha$ , - $\beta$ 2, or - $\beta$ 3 linked to a thrombin cleavage site followed by the hinge, CH2 and CH3 regions of a human IgG1 antibody, with secretion of the proteins directed by the signal sequence of CD5. The EGF-like domain of HRG- $\alpha$  corresponds to

25 residue 177 to 241 of the mature protein, while that of HRG- $\beta$ 2 and - $\beta$ 3 corresponds to residue 177 to 238 and residue 177 to 241, respectively. The three fusion proteins rHRGs-T-Fc are prepared by transient expression in COS cells, purified from

culture supernatants on protein A-Sepharose, and gave yields in the range of 350 to 1900 µg/l. The CD5 signal peptide allowed efficient processing and secretion of the rHRGs-T-Fc. All three fusion proteins are secreted as disulfide-linked homodimers  
5 similar to immunoglobulins and therefore are each capable of presenting two HRG-EGF-like domains. To establish that the rHRGs-T-Fc are able to bind and activate the HER4 receptor, a study is preferably carried out of their potential to induce phosphorylation of HER4 as well as morphological changes and  
10 up-regulation of ICAM-1.

For the production of large amounts of other recombinant growth factors, the chimeric genes of other growth factors such as, epidermal growth factor, transforming growth factor-alpha, amphiregulin, betacellulin, heparin-binding  
15 epidermal growth factor, vaccinia growth factor, cripto, insulin growth factor, insulin-like growth factor, transforming growth factor-beta, platelet-derived growth factor, fibroblast growth factor, and nerve growth factor, can be constructed and expressed in a similar manner as for rHRGs-T-Fc.

20 The activation of the HER4 receptor by rHRGs-T-Fc is examined with CHO/HER4 cells that express high levels of recombinant human p180erbB4/HER4 and have previously been shown to respond to HRG, Plowman, G. D. et al., (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 1746-1750 and Culouscou, J. M. et al.,  
25 (1993) J. Biol. Chem. 268, 18407-18410. rHRG-α-T-Fc, -β2-T-Fc, and -β3-T-Fc, are added to CHO/HER4 cells at 50 and 200 ng/ml for 10 minutes at 37°C. Cells are then lysed and then the pattern of tyrosine phosphorylated proteins are analyzed by anti-

phosphotyrosine Western blotting as compared to untreated cells. As shown in Fig. 1A, all three rHRGs-T-Fc induced the hyper-phosphorylation of the HER4 receptor. Ligand activation not only resulted in receptor autophosphorylation, but also in the tyrosine phosphorylation of several substrates, including a Mr 100,000 band, not identified (Fig. 1A). When tested on CHO/EGFR cells that express high levels of recombinant human EGFR, rHRGs-T-Fc (200 ng/ml) failed to activate the EGFR (Fig. 1B). The EGF (200 ng/ml), markedly induced phosphorylation of the EGFR in CHO/EGFR cells (Fig. 1B, lane 2). These demonstrate that the rHRGs-T-Fc are active molecules and are able to specifically induce HER4 tyrosine phosphorylation.

The binding of rHRG- $\alpha$ -T-Fc to HER4 expressing cells can be shown in the MDA-MB-453 cells that are known to express the HER4 receptor, Plowman, G. D. et al., (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 1746-1750, as well as in the related receptors HER2 and HER3, Kraus, M. H. et al., (1987) EMBO J. 6, 605-610 and Kraus, M. H. et al., 1989) Proc. Natl. Aca. Sci. U.S.A. 86, 9193-9197. These cells are incubated on ice with 1 or 10  $\mu$ g/ml rHRG- $\alpha$ -T-Fc. Bound fusion proteins are detected by adding fluorescein-conjugated anti-human IgG antibodies which recognize the human Fc portion of rHRG- $\alpha$ -T-Fc. As shown in Fig. 2, rHRG- $\alpha$ -T-Fc bound to MDA-MB-453 cells (Fig. 2B, 10  $\mu$ g/mL; and 2D, 1  $\mu$ g/mL). The fluorescence, as analyzed by confocal microscopy, is localized at the periphery of the cells which is consistent with the fact that the staining is performed on live cells kept on ice. When no fusion protein is added, the fluorescein-conjugated anti-human IgG showed no detectable binding to the MDA-MB-453 cells (Fig.

2A). Minimal background staining is observed when an irrelevant Tek-Fc fusion protein is used at 10 µg/ml (Fig. 2C). This result indicates that rHRG-α-T-Fc binds to HER4 expressing cells, and can be used to detect cells expressing HRG binding proteins in a manner similar to monoclonal antibodies. rHRGs-T-Fc represent an alternative to antibodies for cell staining.

NDF, the rat homologue of HRG, has been shown to induce morphological changes in AU565 mammary tumor cells, Peles, E. *et al.*, (1992), *Cell* 69, 205-216, as well as the expression of ICAM-1, Bacus, S. S. *et al.*, (1993), *Cancer Res.* 53, 5251-5261. For carrying out the ability of rHRG-α-T-Fc to induce the expression of ICAM-1 at the surface of MDA-MB-453 cells, these cells are treated for 3 days with 50 ng/ml of the fusion protein and stained with an anti-ICAM-1 antibody. Bound anti-ICAM-1 antibodies are detected using a fluorescein-conjugated anti-mouse IgG antibody. As shown in Fig. 3B, rHRG-α-T-Fc induced a clear up-regulation of ICAM-1 expression in MDA-MB-453 cells as compared to untreated cells (Fig. 3A) and cells treated with an irrelevant Tek-Fc fusion protein (Fig. 3C). p45, a HRG isoform purified from conditioned medium from HepG2 cells, Culouscou, J. M. *et al.*, (1993) *J. Biol. Chem.* 268, 18407-18410, is used at 50 ng/ml as a positive control and induced up-regulation of ICAM-1 (Fig. 3D). The result shows that the rHRGs-T-Fc elicited biological responses similar to those elicited by the natural HRGs in breast carcinoma cells expressing the HER4 receptor and can be used to study the biological consequences of HRG binding to such cells.

The DNA fragments encoding the EGF-like domains of HRGs are amplified by PCR, purified and inserted into a CDM7-

derived expression vector containing sequences encoding a thrombin cleavage site upstream and in frame with the Fc portion of a human IgG1 antibody. The addition of a thrombin cleavage site in the expression vector is based on a system developed by Hakes and Dixon, Hakes, D. J. et al., (1992) Anal. Biochem. 202, 293-298, for recombinant protein expression in bacteria. The presence of a thrombin cleavage site in the rHRGs-T-Fc allows for separation of the two functional domains of the fusion proteins. Following thrombin cleavage, the purified EGF-like domains are recovered as monomeric proteins since the thrombin site is located upstream of the hinge region of the Fc domain of the fusion proteins. rHRG- $\beta$ 2-T-Fc and - $\beta$ 3-T-Fc are incubated with human thrombin. The recombinant EGF-like domains of HRGs (reHRGs) are then separated from the Fc portion of the molecules by protein A-Sepharose chromatography. reHRGs are recovered in the column flow-throughs. Fc portions are recovered from protein A-Sepharose by acid elution. Fig. 4 shows a silver stained polyacrylamide-gel of the rHRG- $\beta$ 3-T-Fc before thrombin cleavage (lane 1). The intact fusion protein displays an apparent molecular mass of 40 kDa under reducing conditions, corresponding to its monomeric form. After cleavage but before protein A-Sepharose (lane 2), a 34 kDa band, corresponding to the Fc portion of the fusion protein, and a 6 kDa band, corresponding to the EGF-like domain of HRG- $\beta$ 3, are identified. The two fragments are separated by protein A-Sepharose chromatography. The 6 kDa EGF-like domain of HRG- $\beta$ 3 (reHRG- $\beta$ 3) is recovered in the column flow-through (lane 3), and the 34 kDa Fc domain of the fusion protein is acid eluted from the column (lane 4).

The stimulation of protein phosphorylation in response to reHRGs are carried out on the purified reHRG- $\beta$ 2, reHRG- $\beta$ 3, and Fc domains of rHRG- $\beta$ 2-T-Fc and rHRG- $\beta$ 3-T-Fc in MDA-MB-453 cells. The intact rHRG- $\beta$ 2-T-Fc and - $\beta$ 3-T-Fc (Fig. 6B, lanes 3 and 6, respectively) are potent stimulators of tyrosine phosphorylation of a 180 kDa protein, as compared to background levels of phosphorylation observed in the absence of treatment (lane 1) or following EGF treatment (lane 2). reHRG- $\beta$ 2 (lane 4) and reHRG- $\beta$ 3 (lane 7) elicited an increase in the phosphorylation level of the 180 kDa protein similar to that obtained with the rHRG- $\beta$ 2-T-Fc and rHRG- $\beta$ 3-T-Fc, whereas the Fc domains from rHRG- $\beta$ 2-T-Fc and rHRG- $\beta$ 3-T-Fc failed to induce protein phosphorylation (lanes 5 and 8, respectively).

Following cleavage of the fusion proteins, several amino acid residues from the glycine rich region of the thrombin cleavage site remain at the carboxy terminus of the reHRGs. Cleavage occurs at the Proline-Arginine recognition sequence of the thrombin cleavage site. See Hakes, D. J. *et al.*, (1992), *Anal. Biochem.* 202, 293-298. These additional residues did not affect the properties of reHRGs. Among the multiple HRG/NDF isoforms, the region proximal to the EGF domain (referred to as the juxtamembrane region in the HRG/NDF precursor forms) can be absent, e.g. HRG- $\beta$ 2/NDF- $\beta$ 2, or comprise up to 26 amino acids, e.g. NDF- $\beta$ 4, Holmes, W. E. *et al.*, (1992), *Science* 256, 1205-1210 and Wen, D. *et al.*, (1994), *Mol. Cell Biol.* 14, 1909-1919. A truncated form of NDF- $\alpha$  that lacks the juxtamembrane region displays the same receptor binding affinity as the full-length NDF- $\alpha$  isoform, implying that this region proximal to the EGF domain is



not involved in receptor binding, Wen, D. et al., (1994), Mol. Cell Biol. 14, 1909-1919. This shows that, after thrombin cleavage, the reHRGs retain the activity displayed by the rHRGs-T-Fc fusion proteins.

- 5           Because the MDA-MB-453 cells express HER3, HER4, and also high levels of HER2, the exact identity of the 180 kDa phosphorylated band is unknown. HER4 and HER3 have both been identified as HRGs receptors, Plowman, G. D. et al., (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1746-1750; Culouscou, J. M. et al., (1993) J. Biol. Chem. 268, 18407-18410; Plowman, G. D. et al., (1993) Nature (London) 366, 473-475 and Carraway, K. L. et al., (1994) J. Biol. Chem. 269, 14303-14306. Since the samples are electrophoresed under reducing conditions, the phosphorylated species corresponds to a combination of
- 10           monomeric forms of all three receptors, including HER2. In response to HRG stimulation, HER2 is stimulated indirectly through receptor transphosphorylation, Plowman, G. D. et al., (1993) Nature (London) 366, 473-475. In addition, it has been indicated that a high affinity binding site for the EGF-like domain of HRG- $\beta$ 1 can be reconstituted by co-expression of HER2 and HER3
- 15           in COS-7 cells, and that binding of HRG results in tyrosine phosphorylation of both HER2 and HER3, Sliwkowski, M. X. et al., (1994) J. Biol. Chem. 269, 14661-14665.
- 20           The results described clearly demonstrate that the EGF-like domains of rHRGs-T-Fc mediate the observed biological effects and that those effects cannot be attributed to the Fc portion of the fusion proteins:
- 25

The phosphorylation of Shc upon HER4 receptor activation is carried out as follows: the activation of receptor tyrosine kinases, such as the EGF receptor, the insulin receptor, and the PDGF receptor results in phosphorylation of a number of intracellular signaling molecules, Ullrich, A. et al., (1990) Cell 61, 203-212 and White, M. F. et al., (1994) J. Biol. Chem. 269, 1-4. For analyzing the molecules that are involved in HRG signaling, MDA-MB-453 cells are stimulated with or without 200 ng/ml reHRG- $\beta$ 2. Cell lysates are immunoprecipitated with the following antibodies: anti-GAP, anti-PLC-g1, anti-P I 3-K, and anti-Shc. Precipitated proteins are separated by SDS-PAGE, then immunoblotted with antiphosphotyrosine antibodies. Shc and, to a lesser degree, P I 3-K immunoprecipitates displayed enhanced patterns of protein phosphorylation following HRG stimulation. A further analysis of Shc phosphorylation was carried out. Shc proteins are ubiquitously expressed proteins containing a single SH2 domain. Three structurally related Shc proteins, p46Shc, p52Shc, and p66Shc have been described as adaptor molecules that are implicated in Ras activation, Pelicci, G. et al., (1992) Cell 70, 93-104 and Rozakis-Adcock et al., (1992) Nature (London) 360, 689-692. CHO/HER4 cells and MDA-MB-453 cells are exposed to reHRG- $\beta$ 2, and lysed. Equivalent amounts of cell lysates are immunoprecipitated with an anti-Shc antibody, and blotted with either anti-Shc (Fig. 6A), or anti-phosphotyrosine antibodies (Fig. 6B). Fig. 6A shows that equal amounts of proteins from stimulated and unstimulated cell lysates are loaded per lane, and that MDA-MB-453 cells (lanes 1 and 2) express only p46Shc and p52Shc (p66Shc is not detected in the assay), whereas

CHO/HER4 cells (lanes 3 and 4) express all three Shc isoforms. p66Shc is translated from a different transcript than the other two Shc isoforms and is not expressed in every cell type, for example it is absent in human hematopoietic cell lines, Pelicci, G. et al.,  
5 (1992) Cell 70, 93 -104. As seen in Fig. 6B, reHRG- $\beta$ 2 induced hyper-phosphorylation of Shc in both cell types. In MDA-MB-453 cells, reHRG- $\beta$ 2 stimulation resulted in tyrosine phosphorylation of both p46Shc and p52Shc (lanes 1 and 2). Following reHRG- $\beta$ 2 stimulation, phosphorylation of p52Shc is markedly increased in  
10 CHO/HER4 cells (lanes 3 and 4). p46Shc appeared to display a relatively high endogenous level of phosphorylation in those cells, and is only marginally affected following HRG treatment. Longer exposure time of the blot shown in Fig. 6B, lanes 3 and 4, resulted in a loss of resolution between the p46Shc and p52Shc bands  
15 but revealed that p66Shc is phosphorylated in response to reHRG- $\beta$ 2 (lane 6) as compared to unstimulated cells (lane 5).

The results presented in the instant invention indicate recombinant EGF-like domains of HRG- $\alpha$ , - $\beta$ 2, and - $\beta$ 3 fused to a thrombin cleavage site followed by the Fc domain of a human IgG1.  
20 These reagents are useful in in vitro assays as fusion proteins or as a source of truncated recombinant HRGs. The results also indicate that both forms in vitro can activate the HER4 receptor and elicit known HRG biological responses. The present invention shows for the first time, that following HRG stimulation, Shc  
25 proteins which have been implicated in Ras activation pathway are phosphorylated on tyrosine. The availability of the recombinant HRGs will allow further experiments to dissect the mechanism of HRG receptor signaling as well as compare the

HER4 substrates to those of other members of the EGFR family of tyrosine kinases.

In order to further clarify and enable the present invention as illustrated in the Examples described herein below,  
5 a general description of the materials and methods utilized in producing the results disclosed is presented. These materials and methods illustrate the technology utilized and are not intended to be limiting of the present invention. Other variations and  
10 modifications of these methods are well-known and are contemplated by this invention.

Antibodies – RC20 recombinant antiphosphotyrosine antibody (Transduction Laboratories) and PY20 antiphosphotyrosine antibody (ICN Biomedicals, Inc.) used in  
15 Western blotting studies were purchased from Transduction Laboratories and ICN Biomedicals, Inc. Polyclonal anti-Shc antibodies were purchased from Upstate Biotechnology Incorporated and the monoclonal anti-Shc antibody was  
20 purchased from Transduction Laboratories. BBA 3, the anti-human ICAM-1 monoclonal antibody, was purchased from R & D Systems.

Cell Lines – MDA-MB-453 human breast cancer cells were obtained from the American Type Culture Collection. CHO/EGFR cells were generated by Dr. B. Thorne (Bristol-Myers Squibb, Seattle, WA.) as follows: the complete recombinant  
25 human EGF receptor coding sequence was inserted into a CDM8 expression vector containing the neomycin resistance gene. The resulting construct was transfected into Chinese hamster ovary cells (CHO-K1). G418 resistant clones were analyzed for EGFR

expression. Levels of expression of functional EGFR in CHO/EGFR stable cells were assessed by stimulating the cells with EGF, immunoprecipitating the EGFR and determining its phosphorylation level by phosphotyrosine Western blotting as reported, by Plowman, G. D. et al., (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 1746-1750. CHO/HER4 cells expressing high levels of recombinant human HER4 have previously been described, by Plowman, G. D. et al., (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 1746-1750; Culouscou, J. M. et al., (1993) J. Biol. Chem. 268, 18407-18410 and Plowman, G. D. et al., (1993) Nature (London) 366, 473-475.

### Example 1

#### CONSTRUCTION OF HRG-T-FC EXPRESSION PLASMIDS

DNA fragments encoding part of the spacer domain of the human HRGs, the EGF-like domains, the transmembrane domain, and a few residues of the cytoplasmic domain were amplified by RT-PCR from total RNA isolated from HepG2 cells. The oligonucleotide primers were designed based on the sequence of the human HRG- $\beta$ , Holmes, W. E. et al., (1992), Science 256, 1205-1210.

The PCR primers used were synthesized and have the following sequences:

5'-GTGTCTTCAGAGTCTCCCATAGA-3' (forward primer, SEQ. I.D. NO.: 1) and

5'-CTTGGTTTTGCAGTAGGCCAC-3' (reverse primer, SEQ. I.D. NO.: 2). Amplification was performed with Taq DNA polymerase (Perkin-Elmer Roche) using 35 cycles, each cycle being composed of a 1 minute at 95°C denaturing step, 1 minute

at 65°C annealing step, and 30 sec at 72°C extension step. The PCR products were blunt-ended using the Klenow fragment of E. coli DNA polymerase I, subcloned into a Sma-1 digested pBluescript II vector (Stratagene) and the nucleotide sequence of individual clones was determined by the dideoxy-mediated chain termination reaction. This procedure generated EGF-like domains of HRG- $\alpha$ , - $\beta$ 2, and - $\beta$ 3

The EGF-like domains of HRG- $\alpha$ , - $\beta$ 2, and - $\beta$ 3 were generated by PCR using HRG- $\alpha$ , or - $\beta$ 2 template plasmids generated as described above. The oligonucleotide primers described below were designed to place a SpeI site at the 5' end and a BamHI site at the 3' end of the amplified products for cloning purposes. The epidermal growth factor-like domain of the human HRG- $\alpha$  was amplified using the following sequences:

5'-GAGACTAGTAGCCATCTTGTAATGTGCG-3' (forward, SEQ. I.D. NO.: 3), and

5'-CCGTGGATCCTTCTGGTACAGCTCCTCCGC-3' (reverse, SEQ. I.D. NO.: 4. PCR conditions consisted of 40 cycles of 30 sec at 94°C, 1 minute at 55°C, and 2 minutes at 72°C, using Pfu polymerase and reagents recommended by the vendor (Stratagene Corp.). The PCR product encoded complementary sequences corresponding to residue 177 to 241 of HRG- $\alpha$ . The epidermal growth factor-like domains of human HRG- $\beta$ 2 and - $\beta$ 3 were amplified using a HRG- $\beta$ 2 clone as a template. The forward primer is shown above (SEQ. I.D. No.: 3). The HRG- $\beta$ 2 reverse primer had the sequence:

5'-CCGTGGATCCTTCTGGTACAGCTCCTCCGCCTT-3' (SEQ. I.D. NO.: 5). Amplification was performed with Pfu

polymerase using the same temperature conditions as that used for HRGa. This PCR product encoded sequences corresponding to residue 177 to 238 of HRG- $\beta$ 2. The HRG- $\beta$ 3 reverse primer contained a silent point mutation introducing a HindIII site for diagnostic purposes and had the following sequence:

5'-CCGTGGATCCTCAGGCAAGCTTAGAAAGGGA  
GTGGACGTACTGTAGAAGCTGGCCATTAC-3'

(SEQ. I.D. NO.:6). PCR conditions consisted of 40 cycles of 1 minute at 94°C, 2 minutes at 50°C, and 3 minutes at 72°C using

Pfu polymerase. The PCR product encoded sequences corresponding to residues 177 to 241 of HRG- $\beta$ 3. All PCR products were digested with BamHI and SpeI and ligated to a BamHI-SpeI-cut CDM7-derived vector containing cDNA sequences coding for the CD5 signal peptide 5' of the cloning site for proper secretion of the expressed proteins, as well as cDNA sequences encoding a thrombin cleavage site (amino acid sequence DPGGGGGRLVPRGFGTG; Sequence I.D. No. 7) and cDNA sequences encoding the hinge and constant regions of a human IgG1, 3' of the cloning site. All constructs were sequenced by the dideoxy-mediated chain termination reaction to confirm the sequence of the EGF-like domains as well as to verify that their sequences were in frame with the thrombin and Fc coding sequences. This procedure resulted in the production of the constructs or the expression plasmids of HRGs-T-Fc. The sequence of a rHRG- $\alpha$ -T-Fc appears in Figures 7A and B.

### Example 2

#### TRANSFECTION OF CONSTRUCTS INTO COS CELLS

The above constructs were transfected into COS cells as previously described, Seed, B. et al., (1987) Proc. Natl. Acad. Sci. USA 87, 3365-3369, and the resulting fusion proteins recovered from culture supernatants using protein A-Sepharose (Repligen). Purified proteins were visualized on 8% SDS-PAGE under reducing and non-reducing conditions. Protein concentrations were determined using a protein assay kit (Bio-Rad Labs). This experiment resulted in the fusion protein, rHRG- $\alpha$ -T-Fc, rHRG- $\beta$ 2-T-Fc or rHRG- $\beta$ 3-T-Fc

### Example 3

#### THROMBIN CLEAVAGE OF FUSION PROTEINS

Fusion proteins were incubated for 30 minutes at room temperature with human thrombin (purchased from Sigma, St. Louis, MO) at a 1:50 (w/w) thrombin: fusion protein ratio. Cleaved proteins were then loaded on a protein A-Sepharose column. Column flow-throughs containing the recombinant EGF-like domain of HRGs were stored at -20°C. This procedure gave recombinant protein reHRG- $\alpha$ , reHRG- $\beta$ 2 or reHRG- $\beta$ 3.

### Example 4

#### DETECTION OF TYROSINE-PHOSPHORYLATED PROTEINS BY WESTERN BLOTTING

CHO/HER4 cells ( $5 \times 10^4$ ), CHO/EGFR cells ( $2 \times 10^4$ ), and MDA-MB-453 cells ( $4 \times 10^5$ ) were seeded in 48-well plates. 24 hours later, cells were serum-starved for 8 hours and then



stimulated with various samples for 10 minutes at 37°C. Supernatants were discarded, and cells were lysed by adding boiling electrophoresis sample buffer. Lysates were subjected to SDS-PAGE on 8% polyacrylamide gels (purchased from Novex) and then electroblotted onto nitrocellulose. PY20 monoclonal anti-phosphotyrosine antibody (purchased from ICN) and horseradish peroxidase-conjugated goat anti-mouse IgG F(ab')<sub>2</sub> (purchased from Cappel) were used as primary and secondary probing reagents, respectively. Immunoreactive bands were visualized using enhanced chemiluminescence (purchased from Amersham Corp.). The results showed the pattern of tyrosine phosphorylated proteins in HER4 receptor which is illustrated in Fig. 1.

15 Example 5  
IMMUNOPRECIPITATION

CHO/HER4 cells were seeded in 100-mm dish. 80-90% confluent monolayers were washed and incubated with the various recombinant HRGs for 10 minutes at 37°C. Monolayers were washed with ice-cold PBS, and solubilized for 10 minutes on ice in PBSTDS lysis buffer (10 mM sodium phosphate, pH 7.3, 150 mM NaCl, 1% Triton-X100, 0.5% sodium deoxycholate, 0.1% SDS) containing 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 20 µg/ml aprotinin, 20 µg/ml leupeptin, 20 µg/ml pepstatin. The protein concentrations of the clarified extracts were determined using a BCA protein assay kit (purchased from Pierce). Lysates (1 mg per immunoprecipitation) were incubated overnight at 4°C with a rabbit anti-Shc antibody (purchased from

UBI). Immune complexes were precipitated by adding protein G-Plus/Protein A-Agarose (purchased from Oncogene Sciences) to the suspensions. After one hour of incubation at 4°C, the immunoprecipitates were washed three times with PBSTDS and then resolved on 8% polyacrylamide gels (purchased from Novex) under reducing conditions. Proteins were electroblotted onto nitrocellulose and probed with RC20 recombinant anti-phosphotyrosine antibody (purchased from Transduction Laboratories) or anti-Shc monoclonal antibody (purchased from Transduction Laboratories). Immunoreactive bands were visualized using enhanced chemiluminescence (purchased from Amersham Corp.). The results showed the precipitation of Shc proteins and that these proteins are phosphorylated in response to protein reHRG- $\beta$ 2.

15

#### Example 6

##### IMMUNOHISTOCHEMICAL STAINING

MDA-MB-453 cells were plated in 8-well borosilicate chambered slides (purchased from Lab-Tek). For receptor binding visualization, after a 48 hour-culture period, the cells were placed on ice for 10 minutes, washed twice with ice-cold binding buffer (DMEM supplemented with 44 mM sodium bicarbonate, 50 mM Bes, pH 7.0, 0.1% bovine serum albumin) and then incubated on ice for 2 hours with rHRG- $\alpha$ -T-Fc, or as a negative control, an irrelevant fusion protein consisting of the extracellular domain of Tek receptor, Dumont, D. J. et al., (1993) Oncogene 8, 1293-1301, fused to the thrombin cleavage site followed by the Fc region of an IgG1 as in rHRGs-T-Fc. The reagents, cloning vector, and

20

25

mammalian cells used to construct and generate the Tek-Fc fusion protein were identical to the ones used to make the rHRGs-T-Fc.

The cells were washed twice and incubated for 45 minutes on ice with a fluorescein-conjugated goat anti-human IgG F(ab')<sub>2</sub>

- 5 (purchased from Tago). The cells were rinsed twice with PBS and fixed for 20 minutes in PBS, 2% formaldehyde. The results showed that the recombinant fusion protein can be used to stain cells that express HER4 receptor.

10 Example 7

ICAM-1 EXPRESSION STUDIES

For ICAM-1 expression studies, after a 24 hour-culture period, the MDA-MB-453 cells were incubated for three days with 50 ng/ml rHRG- $\alpha$ -T-Fc, p45, Culouscou, J. M. et al., (1993) J. Biol.

- 15 Chem. 268, 18407-18410, Tek-Fc fusion protein as a negative control, or culture medium alone. Staining was then performed on live cells. The cells were washed and incubated for 1 hour on ice with an anti-ICAM-1 antibody (purchased from R & D Systems) diluted 1:500 in binding buffer. The cells were washed and  
20 incubated for 45 minutes on ice with a fluorescein-conjugated goat anti-mouse IgG F(ab')<sub>2</sub> (purchased from Tago). The cells were rinsed and fixed as described above. The levels of receptor staining and ICAM-1 expression were analyzed using a Leica confocal microscope. The results showed that fusion protein  
25 induced the expression of ICAM-1 in breast carcinoma cells that express HER4 receptor and is illustrated in Fig. 3.

**Example 8****TYROSINE AUTOPHOSPHORYLATION OF THE HER4  
RECEPTOR****FOLLOWING rHRGS-T-Fc STIMULATION - Figure 1**

5           A. CHO/HER4 cells were incubated in the absence  
(lane 1) or the presence of rHRG- $\alpha$ -T-Fc (lanes 2 and 3), rHRG- $\beta$ 2-  
T-Fc (lanes 4 and 5), and rHRG- $\beta$ 3-T-Fc (lanes 6 and 7) at 50  
ng/ml (lanes 2, 4, and 6) or at 200 ng/ml (lanes 3, 5, and 7). Cells  
were lysed, proteins separated by SDS-PAGE, transferred to  
10 nitrocellulose and immunoblotted with antiphosphotyrosine  
antibodies. Horseradish peroxidase-conjugated goat anti-mouse  
IgG antibodies and chemiluminescence reagents were used to  
visualize the bound antibodies. The results illustrated in Fig. 1A  
showed that all three rHRGs-T-Fc induced the hyper-  
15 phosphorylation of the HER4 receptor. Also, ligand activation  
resulted in receptor autophosphorylation as well in the tyrosine  
phosphorylation of several substrates.

          B. CHO/EGFR cells were incubated in the absence  
(lane 1) or the presence of EGF (lane 2), rHRG- $\alpha$ -T-Fc (lane 3),  
20 rHRG- $\beta$ 2-T-Fc (lane 4), and rHRG- $\beta$ 3-T-Fc (lane 5) used at 200  
ng/ml. Cells lysates were processed as described in A. The  
positions of HER4 and EGFR are indicated. These results  
illustrated in Fig. 1B showed that rHRGs-T-Fc failed to activate the  
EGFR. Fig. 1B (lane 2) showed that EGF markedly induced  
25 phosphorylation of the EGFR in CHO/EGFR cells.

### Example 9

#### BINDING OF rHRG- $\alpha$ -T-Fc TO MDA-MB-453 CELLS - Figure 2

- Cells were plated in 8-well Lab-Tek (Nunc) chamber slides at  $2 \times 10^5$  cells/ well. After 2 days, the cells were placed on ice and stained with rHRG- $\alpha$ -T-Fc at 10  $\mu$ g/ml (panel B) and 1  $\mu$ g/ml (panel D), or with an irrelevant fusion protein used at 10  $\mu$ g/ml (panel C). No fusion proteins were added in the experiment shown in panel A. Fluorescein-labeled goat anti-human Fc antibodies were used to visualize the bound fusion proteins.
- Fluorescent staining was analyzed by confocal microscopy. The results illustrated in Fig. 1A showed no binding to the cells when fusion protein was not added to it. The results illustrated in Fig. 2B showed that rHRG- $\alpha$ -T-Fc bound to MDA-MB-453 cells. The results illustrated in Fig. 2C showed minimal background staining when an irrelevant Tek-Fc fusion protein was used. The results of this experiment indicated that rHRG- $\alpha$ -T-Fc bound to HER4 expressing cells and can be used to detect cells expressing HRG binding proteins:

### Example 10

#### THE INDUCTION OF ICAM-1 EXPRESSION IN RESPONSE TO rHRG- $\alpha$ -T-Fc - Figure 3

- MDA-MB-453 cells were cultured for 24 hours in 8-well Lab-Tek (Nunc) chamber slides. Cells were treated with 50 ng/ml rHRG- $\alpha$ -T-Fc (panel B), irrelevant fusion protein (panel C), p45, Culouscou, J. M. et al., (1993) J. Biol. Chem. 268, 18407-18410, (panel D), or left untreated (panel A). Following three additional days of incubation the cells were stained with an anti-ICAM-1

monoclonal antibody. Fluorescein-labeled goat anti-mouse Fc antibodies were used to visualize the bound anti-ICAM-1 antibodies. Staining was analyzed by confocal microscopy. Fig. 3B showed that rHRG- $\alpha$ -T-Fc induced a clear up-regulation of ICAM-1 expression in MDA-MB-453 cells as compared to untreated cells (Fig. 3A) and cells treated with an irrelevant Tek-Fc fusion protein (Fig. 3C). Fig. 3D showed that p45 induced up-regulation of ICAM-1. Thus, the results of this experiment showed that rHRGs-T-Fc elicited biological responses similar to those elicited by the natural HRGs in breast carcinoma cells expressing the HER4 receptor.

#### Example 11

##### THROMBIN CLEAVAGE OF rHRG- $\beta$ 3-T-FC - Figure 4

The fusion protein was incubated with human thrombin at room temperature for 30 minutes, and loaded on a protein A-Sepharose column. The EGF-like domain of HRG- $\beta$ 3 (reHRG- $\beta$ 3) was recovered in the column flow-through while the Fc portion of the fusion protein was eluted from the column. The resulting products were analyzed by SDS-PAGE, and silver stained. Lane 1, rHRG- $\beta$ 3-T-Fc, untreated; lane 2, rHRG- $\beta$ 3-T-Fc, after thrombin cleavage; lane 3, protein A-Sepharose column flow-through (reHRG- $\beta$ 3); lane 4, protein A-Sepharose column eluate (Fc portion of the fusion protein). Fig. 4 (lane 1) showed a silver stained polyacrylamide gel of the rHRG- $\beta$ 3-T-FC before thrombin cleavage. Fig. 4 (lane 2) showed a 34 kDa band corresponding to the Fc portion of the fusion protein and a 6 kDa band corresponding to the EGF-like domain of HRG- $\beta$ 3. The 6 kDa

EGF-like domain of HrG- $\beta$ 3 (reHRG- $\beta$ 3) was recovered in the column-flow-through (lane 3) and the 34 kDa Fc domain of the fusion protein was acid eluted from the column (lane 4).

5 **Example 12**

**STIMULATION OF PROTEIN PHOSPHORYLATION  
IN RESPONSE TO reHRGS - Figure 5**

MDA-MB-453 cells were incubated in the absence (lane 1) or the presence of EGF (lane 2), rHRG- $\beta$ 2-T-Fc (lane 3),  
10 reHRG- $\beta$ 2 (lane 4), Fc portion of the rHRG- $\beta$ 2-T-Fc fusion protein (lane 5), rHRG- $\beta$ 3-T-Fc (lane 6), reHRG- $\beta$ 3 (lane 7), Fc portion of the rHRG- $\beta$ 3-T-Fc fusion protein (lane 8), at 200 ng/ml. Cells were lysed, proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane and blotted with an anti-phosphotyrosine  
15 antibody. Immunoreactive bands were visualized with enhanced chemiluminescence reagents. The results showed that rHRG- $\beta$ 2-T-Fc and - $\beta$ 3-T-Fc (lanes 3 and 6 respectively) were potent stimulators of tyrosine phosphorylation of 180 kDa protein as compared to background level of phosphorylation observed in the  
20 absence of treatment (lane 1) or following EGF treatment (lane 2). Fig. 5 (lanes 4 and 7) showed that reHRG- $\beta$ 2 and reHRG- $\beta$ 3 elicited an increase in the phosphorylation level of the 180 kDa protein, whereas the Fc domains from rHRG- $\beta$ 2-T-Fc and rHRG- $\beta$ 3-T-Fc failed to induce protein phosphorylation (Fig. 5, lanes 5  
25 and 8).

### Example 13

#### TYROSINE PHOSPHORYLATION OF SHC PROTEINS UPON HER4 ACTIVATION - Figure 6A and 6B

MDA-MB-453 cells (lanes 1 and 2) and CHO/HER4 cells  
5 (lanes 3 to 6) were treated with (+) or without (-) 200 ng/ml of  
reHRG- $\beta$ 2 for 10 minutes at 37°C and solubilized. Cell lysates  
containing equal amounts of protein (1 mg) were precipitated with  
a polyclonal rabbit anti-Shc antibody. Immune complexes were  
washed, separated by SDS-PAGE, and transferred to  
10 nitrocellulose.

- A. Shc proteins were detected by immunoblot using a  
monoclonal anti-Shc antibody.
- B. Tyrosine phosphorylation of Shc proteins was analyzed  
by immunoblot using antiphosphotyrosine antibodies. The  
15 positions of the three Shc isoforms are indicated.

The results illustrated in Fig. 6A showed that MDA-MB-  
453 cells (lanes 1 and 2) express only p46Shc and p52Shc  
whereas CHO/HER4 cells (lanes 3 and 4) express all three Shc  
isoforms. Fig. 6B showed that reHRG- $\beta$ 2 induced hyper-  
20 phosphorylation of Shc in both cell types. In MDA-MB-453 cells,  
reHRG- $\beta$ 2 stimulation resulted in tyrosine phosphorylation of both  
p46Shc and p52Shc (lanes 1 and 2). Following reHRG- $\beta$ 2  
stimulation, phosphorylation of p66Shc was markedly increased in  
CHO/HER4 cells (lanes 3 and 4). Longer exposure time of the  
25 blot (Fig. 6B, lane 3 and 4) resulted in a loss of resolution between  
the p46Shc and p52Shc bands, but revealed that p66Shc was  
phosphorylated in response to reHRG- $\beta$ 2 (lane 6) as compared to  
unstimulated cells (lane 5).



Example 14CONSTRUCTION OF EXPRESSION PLASMIDS  
OF OTHER GROWTH FACTORS

- 5           The expression plasmids of other growth factors can be constructed analogously from cDNA's of other growth factors, such as epidermal growth factor, transforming growth factor-alpha, amphiregulin, betacellulin, heparin-binding epidermal growth factor, vaccinia growth factor, cripto, insulin growth factor, insulin-like growth factor, transforming growth factor-beta, platelet-derived growth factor, fibroblast growth factor, or nerve growth factor, according to Example 1 and expressed according to Example 2.
- 10

The foregoing description and Examples are intended as illustrative of the present invention, but not as limiting.

- 15       Numerous variations and modifications may be effected without departing from the true spirit and scope of the present invention.

What We Claim Is:

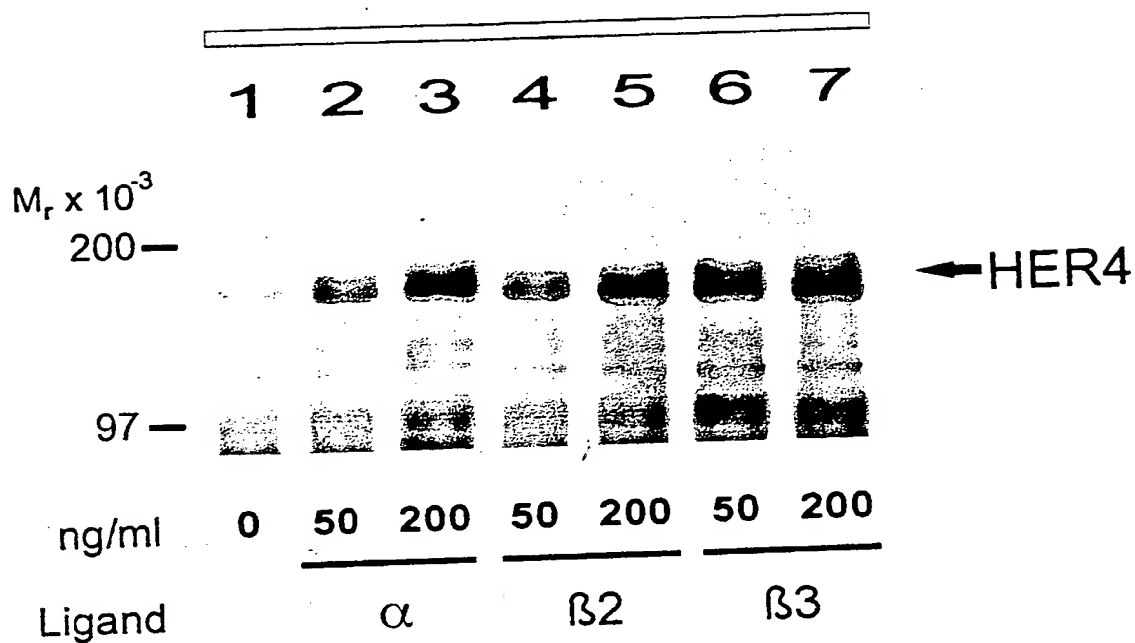
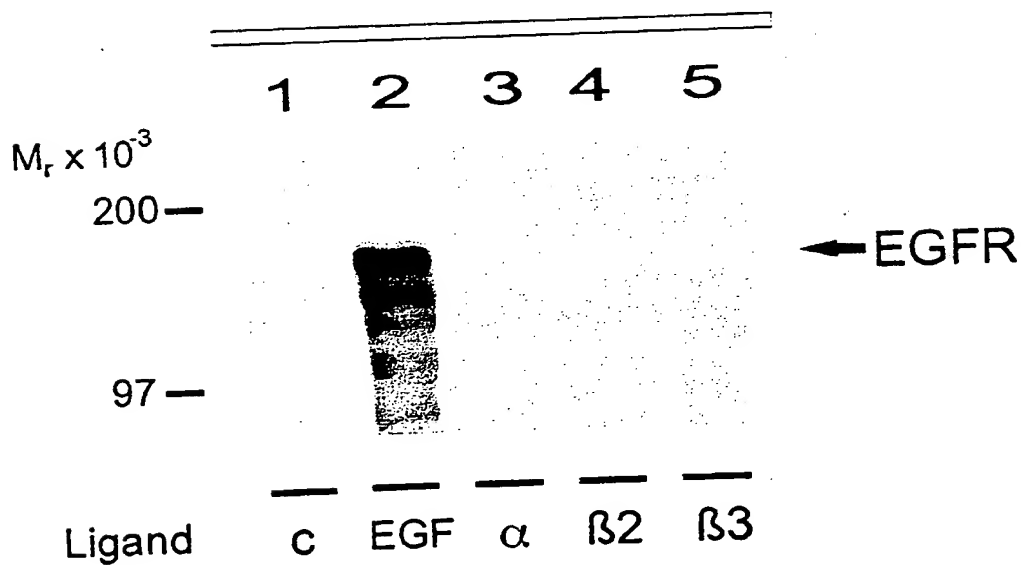
1. A eukaryotic vector comprising cDNA encoding:
  - a. a growth factor domain;
  - 5 b. a thrombin cleavage site; and
  - c. the Fc portion of a human IgG1 antibody.
2. The vector of claim 1, wherein the growth factor is heregulin, epidermal growth factor, transforming growth factor-  
10 alpha, amphiregulin, betacellulin, heparin-binding epidermal growth factor, vaccinia growth factor, cripto, insulin growth factor, insulin-like growth factor, transforming growth factor-beta, platelet-derived growth factor, fibroblast growth factor, or nerve growth factor.
- 15 3. The vector of claim 1, wherein the growth factor domain is the epidermal growth factor-like domain of heregulin- $\alpha$ , - $\beta$ 2 or - $\beta$ 3.
- 20 4. The vector of claim 3, wherein the cDNA encoding the epidermal growth factor-like domain of heregulin- $\alpha$ , - $\beta$ 2 or - $\beta$ 3 is in frame with the thrombin cleavage site and the cDNA sequence encoding the Fc portion of a human IgG1 antibody.
- 25 5. The vector of claim 3, further comprising a cDNA sequence encoding a CD5 signal peptide located 5' of the cloning site.

6. The vector of claim 3, wherein the cDNA sequence encoding the Fc portion encodes the hinge, CH2 and CH3 regions of a human IgG1 antibody.
- 5 7. The vector of claim 5, which is CDM7-derived.
8. A host cell comprising the vector of claim 5.
9. The host cell of claim 8, wherein the host cell is a  
10 mammalian cell.
10. The host cell of claim 9 wherein the mammalian cell is a COS cell.
- 15 11. A process for producing a fusion protein comprising the epidermal growth factor-like domain of heregulin- $\alpha$ , - $\beta$ 2 or - $\beta$ 3 and the Fc portion of a human IgG1 antibody, wherein the process comprises:
- 20 a. transfecting the vector of claim 5 into COS cells under conditions permitting the production of the fusion protein and
- b. recovering the fusion protein produced thereby.
12. The process of claim 11, wherein the fusion protein  
25 recovered from the culture supernatants is purified in a single step using protein A-Sepharose.

13. The process of claim 12, wherein the fusion protein recovered is rHRG- $\alpha$ -T-Fc, rHRG- $\beta$ 2-T-Fc, or rHRG- $\beta$ 3-T-Fc.
14. A process for producing a recombinant protein reHRG- $\alpha$ ,  
5 reHRG- $\beta$ 2-or reHRG- $\beta$ 3, comprising :  
a. cleaving a fusion protein rHRG- $\alpha$ -T-Fc, rHRG- $\beta$ 2-T-Fc or rHRG- $\beta$ 3-T-Fc with human thrombin, and  
b. recovering the protein reHRG- $\alpha$ , reHRG- $\beta$ 2 or reHRG- $\beta$ 3 produced thereby.
- 10 15. A process for producing a fusion protein comprising the epidermal growth factor-like domain of heregulin- $\alpha$ , - $\beta$ 2 or - $\beta$ 3 and the Fc portion of a human IgG1 antibody, wherein the process comprises:  
15 a. transfecting the vector of claim 1 into COS cells under conditions permitting the production of the fusion protein and  
b. recovering the fusion protein produced thereby.
- 20 16. A process for producing a fusion protein comprising the epidermal growth factor-like domain of heregulin- $\alpha$ , - $\beta$ 2 or - $\beta$ 3 and the Fc portion of a human IgG1 antibody, wherein the process comprises:  
25 a. transfecting the vector of claim 3 into COS cells under conditions permitting the production of the fusion protein and  
b. recovering the fusion protein produced thereby.

17. The recombinant fusion protein rHRG- $\alpha$ -T-Fc, rHRG- $\beta$ 2-T-Fc or rHRG- $\beta$ 3-T-Fc.
18. A process for identifying cells expressing heregulin  
5 binding proteins, wherein the process comprises:
- a. incubating test cells with the fusion protein of Claim 17; and
  - b. detecting the fusion protein bound to the cells.
- 10 19. The process of claim 18, wherein the cells used are MDA-MB-453 expressing human epidermal growth factor receptor  
4.
- 15 20. The recombinant protein reHRG- $\alpha$ , reHRG- $\beta$ 2 or reHRG- $\beta$ 3.

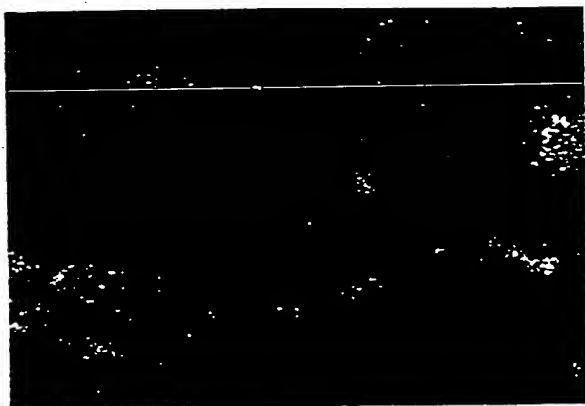
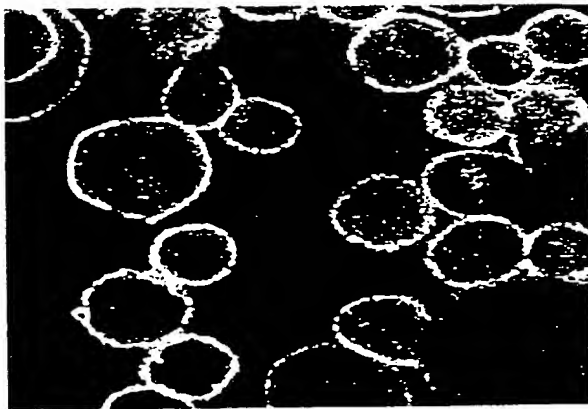
1/7

**CHO/HER4 cells****FIG.1A****CHO/EGFR cells****FIG.1B**

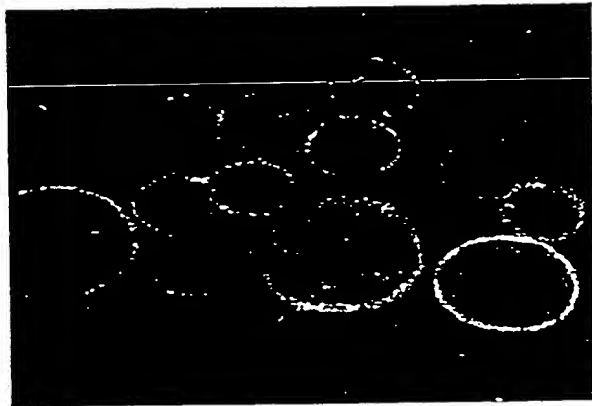
**FIG.2A**



**FIG.2B**



**FIG.2C**



**FIG.2D**

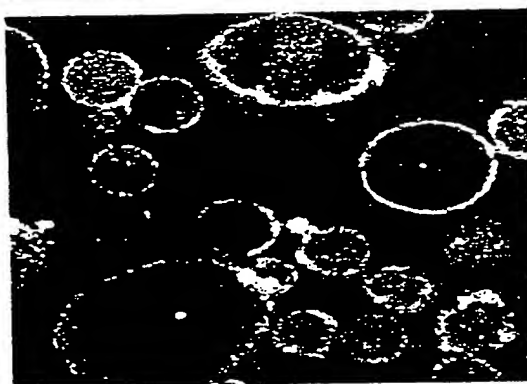
**FIG.3A**



**FIG.3B**



**FIG.3C**



**FIG.3D**



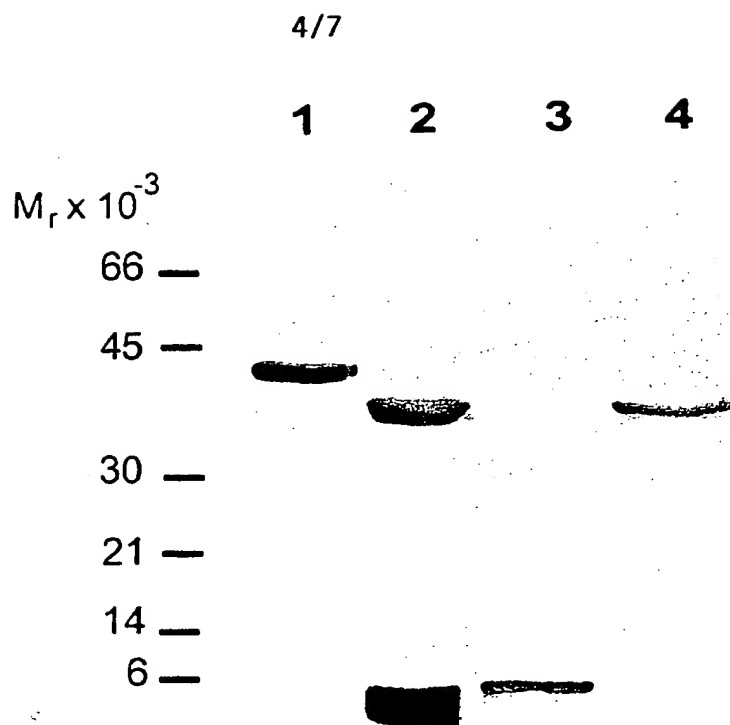


FIG.4

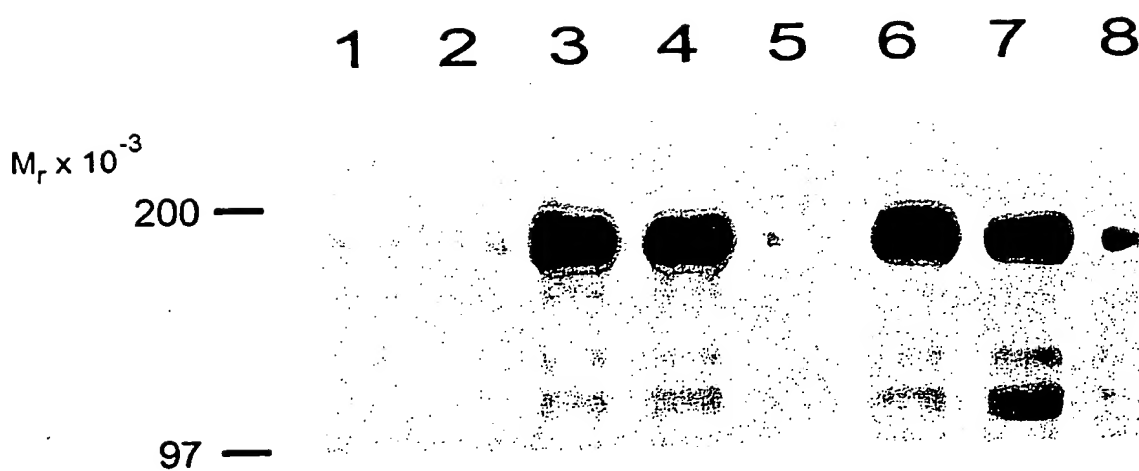
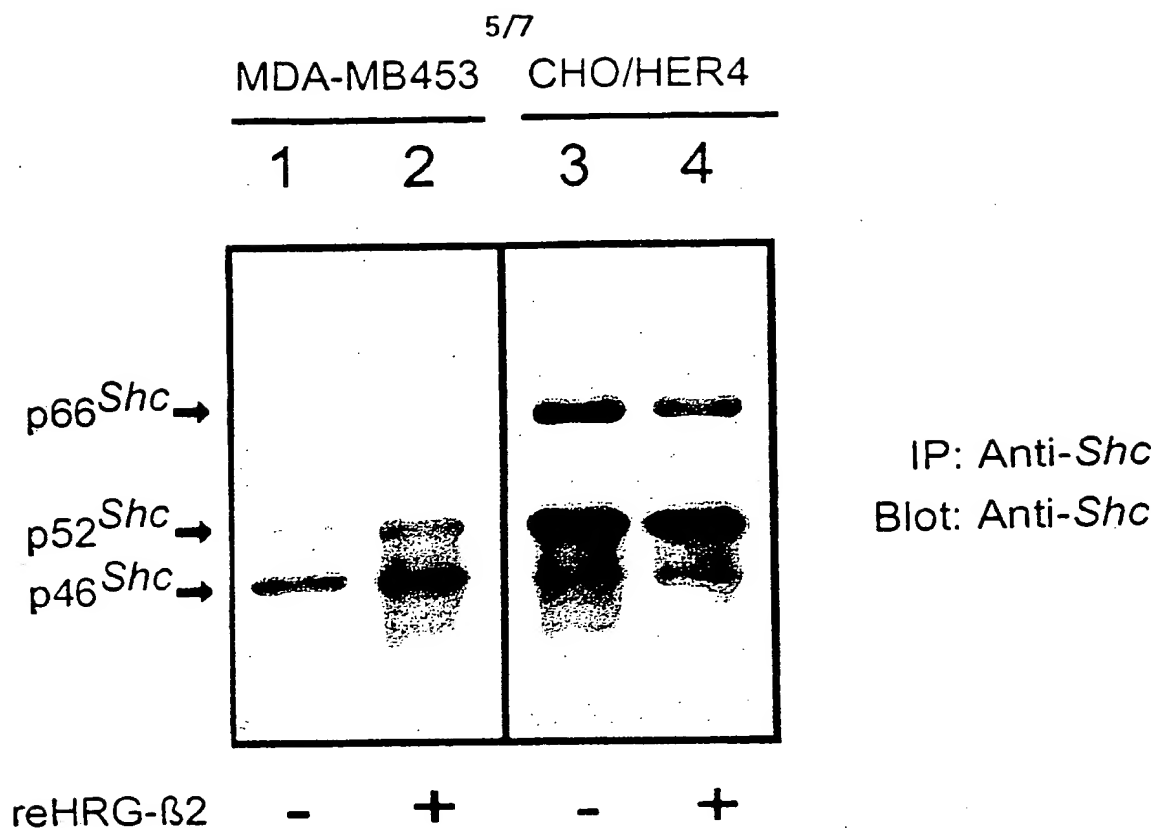
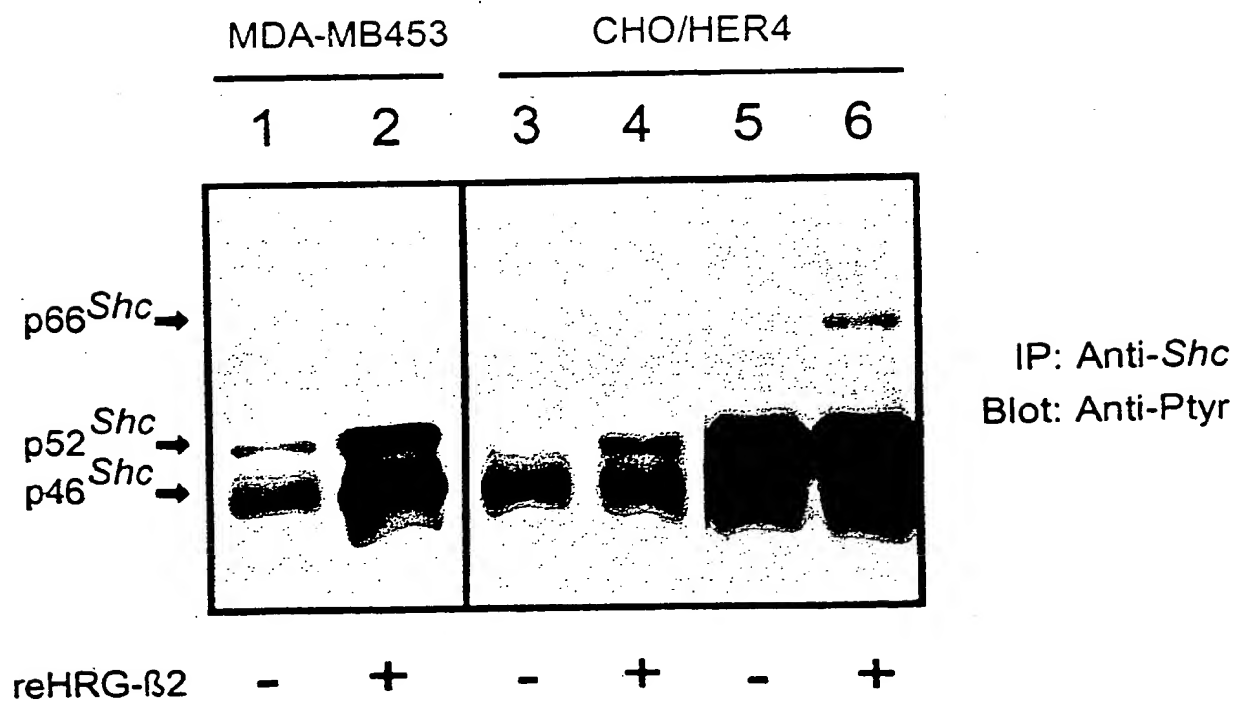


FIG.5

**FIG.6A****FIG.6B**

## FIGURE 7A

aagcttcgagatccattgtgctcctaaaggagatacccgccagacaccctcacctgcggt  
1 -----+-----+-----+-----+-----+ 60  
ttcgaagctctaggttaacacgagatttcctctatgggcccgggtctgtgggagtggaaccca

b S F E I H C A L K E I P G Q T P S P A V -

gcccagctgcccaggctgaggcaagagaaggccagaaaccatgcccattgggtctctgca  
61 -----+-----+-----+-----+-----+ 120  
cgggtcgacgggtccgactccgttctcttcgggtcttttggtacgggtaccccagagacgt

b P S C P G \* G K R R P E T M P M G S L Q -

accgctggccaccttgtacctgctggggatgctggctcgttccctgcctcggaactagtag  
121 -----+-----+-----+-----+-----+ 180  
tggcgaccgggtggaacatggacgacccctacgaccagcgaaggacggagccttgatcatc

b P L A T L Y L L G M L V A S C L G T S S -

ccatcttgtaaaatgtgcgaggagaaggagaaaactttctgtgtgaatggaggggagtgctt  
181 -----+-----+-----+-----+-----+ 240  
ggtagaacattttacacgcctcttctcttttgaaagacacacttacctccccctcacgaa

b H L V K C A E K E K T F C V N G G E C F -

catggtgaaagacctttcaaacccctcgagatacttgtgcaagtgccaacctggattcac  
241 -----+-----+-----+-----+-----+ 300  
gtaccactttctggaaagtgtggggagctctatgaacacgttcacggttggaacctaatg

b M V K D L S N P S R Y L C K C Q P G F T -

tggagcaagatgtactgagaatgtgcccattgaaagtccaaaacccaagaaaaggcggagca  
301 -----+-----+-----+-----+-----+ 360  
acctcgttctacatgactcttacacgggtactttcaggttttggttcttttccgcctcgt

b G A R C T E N V P M K V Q N Q E K A E Q -

gctgtaccagaaggatcccgccggcgccggcgccgcgcctgggttccctcgtggcttcggtag  
361 -----+-----+-----+-----+-----+ 420  
cgacatggtcttcttagggccgcccgcgcgcggcgccggaaccaaggagcaccgaagccatc

L Y Q K D P G G G G G R L V P R G F G S -

cggagatccggagcccaaactcttgtagacaaaactcacacatgcccaccgtgcccagcacc  
421 -----+-----+-----+-----+-----+ 480  
gcctctaggcctcgggttttagaacactgttttgagtggtgtacgggtggcaggggtcgtgg

b G D P E P K S C D K T H T C P P C P A P -

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481 -----+-----+-----+-----+-----+ 540  
acttaagctcccacgtggcagtcagaaggagaaggggggttttggttctctgtgggagta

b E F E G A P S V F L F P P K P K D T L M -

gatctcccggaccctgaggtcacatgcgtgggtgggtggacgtgagccacgaagaccctga  
541 -----+-----+-----+-----+-----+ 600  
ctagagggcctggggactccagtgtagcaccaccacctgcactcgggtgcttctgggact

b I S R T P E V T C V V V D V S H E D P E -

## FIGURE 7B

601 ggtcaagttcaactggtacgtggacggcggtggaggtgcataatgccaaagacaaagccgcg 660  
-----+-----+-----+-----+-----+-----+  
ccagttcaagttgaccatgcacctgccgcacctccacgtattacggttctgtttcggcgc

b V K F N W Y V D G V E V H N A K T K P R -

661 ggaggagcagtacaacagcacgtaccgggtggtcagcgtcctcacgtcctgcaccagga 720  
-----+-----+-----+-----+-----+-----+  
cctcctcgtcatgttgtcgtgcatggcccaccagtcgcaggagtgaggagcgtggtcct

b E E Q Y N S T Y R V V S V L T V L H Q D -

721 ctggctgaatggcaaggagtacaagtgcagggtctccaacaaagccctcccagccccat 780  
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gaccgacttacgttctcctcatgttcacgttccagaggtgtttcgggagggtcgggggta

b W L N G K E Y K C K V S N K A L P A P I -

781 cgagaaaaccatctccaaagccaaagggcagccccgagaaccacaggtgtacaccctgcc 840  
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gctcttttggtagaggtttcgggtttcccgctcggggctcttggtgtccacatgtgggacgg

841 cccatccccgggatgagctgaccaagaaccaggtcagcctgacctgcctgggtcaaaggctt 900  
-----+-----+-----+-----+-----+-----+  
gggtagggccctactcgactggttcttggtccagtcggactggacggaccagtttccgaa

b P S R D E L T K N Q V S L T C L V K G F -

901 ctatcccagcgacatcgccgtggagtgaggagcaatgggcagccggagaacaactacaa 960  
-----+-----+-----+-----+-----+-----+  
gatagggtcgtgtagcggcacctcaccctctcgttaccgctcggcctcttgttgatgtt

b Y P S D I A V E W E S N G Q P E N N Y K -

961 gaccacgcctcccgtgctggactccgacgggtccttcttctctacagcaagctcacgt 1020  
-----+-----+-----+-----+-----+-----+  
ctggtgctggagggcacgacctgaggctgccgaggaagaaggagatgtcgttcgagtggca

b T T P P V L D S D G S F F L Y S K L T V -

1021 ggacaagagcaggtggcagcaggggaacgtcttctcatgctccgtgatgcatgaggctct 1080  
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b D K S R W Q Q G N V F S C S V M H E A L -

1081 gcacaaccactacacgcagaagagcctctccctgtctccgggtaaatga 1129  
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cgtgttggtgatgtgcgtcttctcggagagggacagaggccatttact

b H N H Y T Q K S L S L S P G K \* -

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/06861

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/62 C12N15/12 C07K14/475 A61K38/18

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCIENCE, vol. 256, 22 May 1992, LANCASTER, PA US, pages 1205-1210, XP002015659 W.E.HOLMES ET AL.: "Identification of heregulin, a specific activator of p185erbB2" cited in the application see page 1209, left-hand column, line 15 - line 24	20
Y	CELL, vol. 61, 29 June 1990, NA US, pages 1303-1313, XP002015660 A.ARUFFO ET AL.: "CD44 is the principal cell surface receptor for hyaluronate"	1,2
A	see page 1310: CD5-IgG1 Vector Construction	3-10

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

11 October 1996

Date of mailing of the international search report

23.10.96

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP,A,0 329 175 (TOSOH CORPORATION) 23 August 1989 see page 3, line 4 - line 10 ---	1,2
P,X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 21, 26 May 1995, MD US, pages 12857-12863, XP002015661 J-M CULOUSCOU ET AL.: "HER4 receptor activation and phosphorylation of Shc proteins by recombinant heregulin-Fc fusion proteins" see the whole document -----	1-20

Information on patent family members

PCT/US 96/06861

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